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NOTES

Transfer of the Virulence Plasmid of *Yersinia pestis* to *Yersinia pseudotuberculosis*

HANS WOLF-WATZ,[1]* DANIEL A. PORTNOY,[2]† INGRID BÖLIN,[1] AND STANLEY FALKOW[2]

Division of Microbiology, National Defence Research Institute (FOA 4), S-901 82 Umeå, Sweden,[1] and Department of Microbiology, School of Medicine, Stanford University, Stanford, California 94305[2]

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Transposon Tn*5* insertion derivatives of the virulence plasmid pYV019 of *Yersinia pestis* were transferred by P1 transduction into a plasmid-free strain of *Y. pseudotuberculosis*. One of these plasmid derivatives conferred virulence upon the *Y. pseudotuberculosis* strain. This strain had the ability to express temperature-inducible plasmid-coded outer membrane proteins and was also found to be Ca^{2+} dependent.

All three pathogenic species of yersiniae, *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, possess a related plasmid species which is essential for virulence as well as Ca^{2+}-dependent growth at 37°C in vitro (Ca^{2+} dependence) (1, 6–9, 11–15). We have previously shown that in each *Yersinia* species the plasmid encodes a set of temperature-inducible outer membrane proteins (YOPs) (3–5, 13, 14). *Y. enterocolitica* and *Y. pseudotuberculosis* express these proteins at 37°C in vitro as well as in vivo, whereas *Y. pestis* only expresses the proteins in vivo (4, 10, 13, 14). However, synthesis of YOPs can be detected when the virulence plasmid of *Y. pestis* is transferred to a minicell-producing strain of *Escherichia coli*, showing that this plasmid also has the coding capacity for these proteins (14).

In a recent report it was shown by using transposon Tn*5* that a fairly large region (Ca^{2+} region) of the virulence plasmid pYV019 of the *Y. pestis* EV76 is involved in the Ca^{2+}-dependent behavior (11). It was also shown that there was a strict correlation between Ca^{2+} dependence and virulence as measured by subcutaneous injection of mice, i.e., all transposon Tn*5*-derived, Ca^{2+}-independent, insertion mutants were found to be avirulent (11). The Ca^{2+} region had earlier been shown to be conserved in all three *Yersinia* species, although the virulence plasmid of *Y. enterocolitica* differs by about 50% from the virulence plasmids of *Y. pestis* and *Y. pseudotuberculosis* (14). We report the transfer of the various Tn*5*-derived insertion mutants of plasmid pYV019 (pYV019::Tn*5*) of *Y. pestis* EV76 into the plasmid-free avirulent strain YPIII of *Y. pseudotuberculosis*.

Eight different, previously described, pYV019::Tn*5* plasmids (11) (Fig. 1) were transduced by bacteriophage P1 into the plasmid-free strain YPIII. P1-transduction was performed as described previously (11) with the modification that YPIII was heated to 49°C for 20 min before transduction. This step was presumably necessary to inactivate host restriction enzymes. Transductants carrying pYV019::Tn*5* derivatives were obtained at a rather high frequency after kanamycin selection.

The transductants were analyzed for Ca^{2+} dependence on magnesium oxalate agar plates, as previously described (7, 9). The expression of YOPs was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane preparations obtained from the various transductants after incubation at 37°C as described (14). The virulence of these transductants was established by oral infection of Swiss albino mice (8). The results of these experiments are summarized in Table 1. All plasmids obtained from the resulting transductants were analyzed by restriction enzyme digestion with *Bam*HI. No alterations in plasmid size or restriction pattern was observed when these plasmids were compared with their counterparts, which are described elsewhere (11).

One transductant, YPIII-P4, was found to be fully virulent for mice in contrast to the recipient strain YPIII (Table 1). This clearly shows that plasmid pYV019 contains genetic information corresponding to the wild-type plasmid pIB1 of *Y. pseudotuberculosis* with respect to virulence in mice. This transductant showed the ability to express the temperature-inducible outer membrane proteins YOP2, -4, and -5 (Fig. 2). This result confirmed earlier findings, indicating that plasmid pYV019 has the coding capacity for these proteins although *Y. pestis* does not express them in vitro (14). Furthermore, strain YPIII-P4 showed Ca^{2+} dependency when tested on magnesium oxalate agar plates (Table 1). These results are not surprising considering the high degree of relatedness observed between plasmid pYV019 and pIB1 (14).

Strain YPIII-P3 was also virulent and expressed YOPs to the same extent as the wild-type strain YPIII(pIB1) (Table 1; Fig. 2). However, this pathogen was Ca^{2+} independent. One possible explanation for this finding is that strain YPIII-P3 underwent genetic rearrangements during the process of infection and consequently regained Ca^{2+} dependence. Single-cell, kanamycin-resistant progeny, isolated from the livers and spleens of moribund mice orally infected with strain YPIII-P3, were also Ca^{2+} independent. These isolates caused a fatal infection in mice after oral challange. Thus, Koch's postulates were fulfilled and it can be concluded that Ca^{2+} dependency is not an absolute requirement for expression of virulence in *Y. pseudotuberculosis*.

* Corresponding author.
† Present address: Laboratory of Cellular Physiology and Immunology, Rockefeller University, New York, NY 10021.

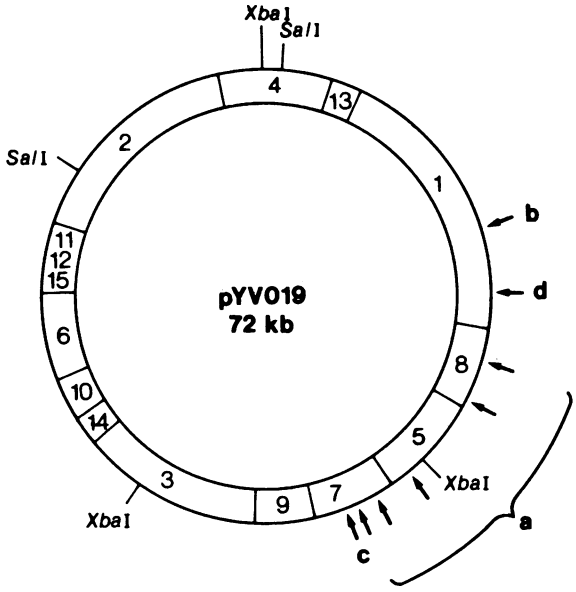

FIG. 1. Genetic location of the different transposon Tn*5* insertions of plasmid pYV019 used in this study. The restriction map and the mapping of the transposon Tn*5* insertions has been described previously (11). The numbered restriction fragments are derived from a *Bam*HI digest of the plasmid. Arrows indicate the position of the different Tn*5* insertions. The nomenclature used is in accordance with that described by Portnoy et al. (11).

Transductant YPIII-P2 was Ca^{2+} dependent but avirulent (Table 1). The only detectable difference, when compared with strain YPIII-P4, was a significant decrease in the amount of YOP4 (molecular weight, 34,000 [Fig. 2]), suggesting that transposon Tn*5* had been inserted either into a structural or a regulatory gene of YOP4. However, it cannot be concluded from analysis of strain YPIII-P2 that YOP4 is a major virulence determinant because this strain was temperature sensitive for growth when shifted from growth at 26 to 37°C, even when Ca^{2+} was added to the culture medium. After prolonged incubation at 37°C in liquid culture, the growth rate declined, followed by a decrease in viability. This phenomenon may explain the avirulent behavior of strain YPIII-P2. Further investigation may reveal mechanisms responsible for the temperature-inducible Ca^{2+} response. When the identical plasmid pYV019::Tn*5*(b) was transduced into *Y. pestis* EV76-6, the resulting transductant was virulent (11), suggesting that YOP4 is not a major virulence determinant for *Y. pestis*.

TABLE 1. Phenotypic behavior of *Y. pseudotuberculosis* harboring plasmid pIB1 or transposon Tn*5* derivatives of plasmid pYV019

Y. pseudotuberculosis	Relevant plasmid description	Ca^{2+} dependence[a]	Virulence[b]
YPIII		−	0/10
YPIII(pIB1) wild type	pIB1	+	10/10
YPIII-P1	pYV019::Tn*5*(a)	−	0/10
YPIII-P2	pYV019::Tn*5*(b)	+	0/10
YPIII-P3	pYV019::Tn*5*(c)	−	10/10
YPIII-P4	pYV019::Tn*5*(d)	+	10/10

[a] Ca^{2+} dependence was determined from viable counts on Mg^{2+} oxalate plates at 37°C as described elsewhere (9).

[b] Virulence was determined by oral challenge of 10 Swiss albino mice as has been described elsewhere (8); 10/10, all mice died; 0/10, all survived.

Five of the transductants represented in Table 1 by strain YPIII-P1, were found to be Ca^{2+} independent, avirulent for mice, and unable to express the YOPs when grown in vitro. These results agree with our earlier results and indicate a correlation between virulence, Ca^{2+} dependence, and expression of the YOPs (4). However, the results reported for strain YPIII-P3, indicate that expression of Ca^{2+} dependence per se is not an essential determinant of pathogenicity.

Two of the strains, YPIII-P3 and YPIII-P4, did not show the expected phenotypes with respect to the Ca^{2+} response,

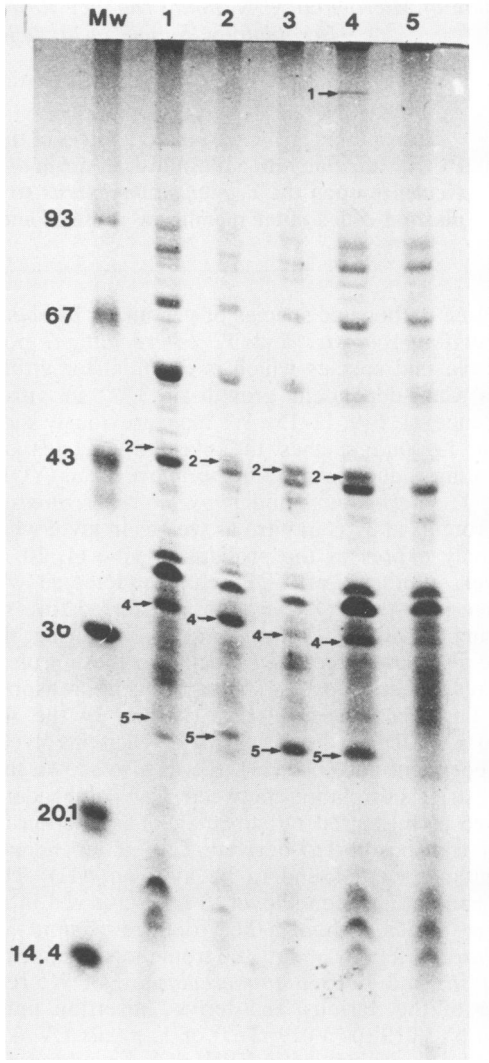

FIG. 2. Membrane protein profiles of different plasmid-containing derivatives of strain YPIII. Different pYV019::Tn*5* plasmids were inserted into strain YPIII by transduction using the phage P1 as described in the text (11). The resulting strains were grown at 37°C in 10 ml of Spizizen minimal medium supplemented with 0.2% glucose, 2% Casamino Acids, and 0.5% yeast extract (10) and incubated overnight. The cells were harvested, the outer membrane was prepared (5), and the membrane protein profile of respective strain was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4). Lanes: 1, YPIII-P3; 2, YPIII-P4; 3, YPIII-P2; 4, YPIII(pIB1); and 5, YPIII. The apparent YOP is labeled with an arrow and a number. Molecular weights (MW) YOP1, 150,000; YOP2, 44,000; YOP4, 34,000; and YOP5, 26,000. YOP3 with a molecular weight of about 40,000 is usually only seen in Western immunoblots, using hyperimmune serum.

based on results obtained in the original studies performed on their plasmids in *Y. pestis* (11). Therefore, we transduced plasmids pYV019::Tn*5*(c) and pYV019::Tn*5*(d) to *Y. pestis* EV76-6. These two transductants now gave phenotypes identical to the corresponding *Y. pseudotuberculosis* strains (Table 1). The reason for the discrepancy is at present unknown, but one possible explanation is that the plasmids were undetectably altered during or subsequent to P1 transduction.

In conclusion, it is clear that the virulence plasmid of *Y. pestis* can be introduced into a plasmid-free avirulent strain of *Y. pseudotuberculosis* and that the novel strain is pathogenic as well as capable of expressing YOPs. These results are in accordance with the close relationship found between strains of *Y. pestis* and *Y. pseudotuberculosis*, which show about 90% sequence homology at the DNA level (2).

It is also obvious that the virulence plasmid pYV019 of *Y. pestis* has the coding capacity for YOPs, and when this plasmid is introduced into a strain with the proper genetic background, these proteins can be expressed in vitro. This indicates that there are chromosomal functions involved in the regulatory expression of the YOPs that might differ between strains of *Y. pestis* and *Y. pseudotuberculosis*.

Although, there seems to be a high correlation between Ca^{2+} dependence and virulence, the Ca^{2+}-dependent behavior is not strictly correlated to either virulence or the expression of YOPs. The ability to express YOPs, however, seems to be one characteristic that is strongly coupled to virulence, since we have so far been unsuccessful in obtaining mutants which separate virulence from expression of the YOPs. More work is needed to establish the possible role of YOPs in the virulence process.

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